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Ontogeny of osmoregulation in the brackishwater amphipod *Gammarus chevreuxi*

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Abstract

Osmoregulation is a key regulatory function in animals inhabiting brackish waters or areas subject to considerable salinity change, such as estuaries. While our understanding of osmoregulation in adult crustaceans is relatively good, our knowledge of how osmoregulatory ability develops during ontogeny is not well documented. In indirect developers, improvement in osmoregulatory capacity during ontogeny appears to coincide with a major metamorphosis. This is consistent with the 'incomplete adult hypothesis', which assumes that early developmental stages are 'incomplete individuals' operating less efficiently than individuals at the older stages. Evidence for this is not clear in direct developers. Consequently, we tested the 'incomplete adult hypothesis', by characterising the ontogeny of osmoregulation of the euryhaline amphipod, *Gammarus chevreuxi*, a species which undergoes direct development. We investigated the structure and function of putative osmoregulatory tissues, together with the regulation of key osmoregulatory genes. Embryos were examined at key developmental stages: before the dorsal organ (DO), a putative osmoregulatory structure, appeared (<48 hpf), before the gills appeared but the DO was present (9 dpf), and after both DO and gills were present (14-18 dpf). Adult *G. chevreuxi* exhibited a pronounced hyper-hypo-osmoregulatory pattern, matched by a strong pattern of haemolymph ion regulation. At a salinity of 35, eggs were hyposmotic to the external medium (989 mOsm Kg^{-1}) in the DO and gill stages (mean±SD= 584±80.5 and 744±103 mOsm Kg^{-1} respectively) with less of a difference with the medium before DO development (mean±SD= 810±91 mOsm Kg^{-1}). At a salinity of 2, eggs from all stages were hyperosmotic to the external medium (52 mOsm Kg^{-1}), with the pre-DO stage being closest to the isosmotic line

(mean±SD= 330±29, 510±55 and 502±55 mOsm Kg^{-1} for pre-DO, DO and gill respectively). Differences between the stages diminished at salinity 15. Thus, the adult hyper-hypo-osmoregulatory pattern was present before the ontogeny of the DO and gills, although it improved during ontogeny. Expression of Na^+/K^+-ATPase transcripts was detected throughout ontogeny, further supporting the idea that ion transporting activity may occur before the formation of osmoregulatory organs. The ontogeny of osmoregulatory function in the gammaridean amphipod *G. chevreuxi* is therefore consistent with the incomplete adult hypothesis.

Key words: Crustacea; Development; Dorsal organ; Embryo; Hyper-hypo-osmoregulation; Na^+,K^+-ATPase

Introduction

Understanding the ontogeny of physiological function and regulation is essential to predicting how and when selection pressures may operate on a developing individual in any given, or changing, environment (Adolph, 1968; Burggren, 2018; Burggren and Warburton, 2005). Such understanding is important in predicting survival through to the reproductively-active adult stage, and consequently Darwinian fitness (Mueller et al., 2015; Spicer et al., 2018). Burggren (2005) suggested that the focus on adult stages in ecophysiological studies is underpinned by the assumption that increasing anatomical complexity invariably leads to increasing physiological complexity. In this view, the early developmental stages would be seen as incomplete individuals operating less efficiently than individuals at the older stages, the so called 'incomplete adult hypothesis' (Adolph, 1968; Spicer and Gaston, 1999). According to this hypothesis, adults would display the most well-regulated physiological processes. The corollary of this is the 'physiological competency hypothesis', which states that 'at every stage, the complement of properties and regulations is complete…for operation of the body' (Adolph, 1968). In this case, the most complex or best-developed physiological regulations or functions would not always be expected to be restricted to, or even present in, the adult stages (Spicer and Gaston, 1999)

Osmoregulation is an important regulatory function that many aquatic animals perform, particularly if they inhabit brackish waters or areas subject to considerable salinity change, such as estuaries (Beadle, 1957; Charmantier et al., 2008; Gilles, 1975; Krogh, 1939; Potts and Parry, 1964). Coastal and estuarine crustaceans have received considerable attention in this regard, and tend to show a strong osmoregulatory ability, at least in the adult stage (Lignot and Charmantier, 2015;

Lockwood, 1962; Rivera-Ingraham and Lignot, 2017; Robertson et al., 2010; Schoffeniels and E., 1970; Thabet et al., 2017). Such osmoregulatory functions are carried out by combinations of the following structures: the gills, the gut, the antennal gland, and extrabranchial ion exchange tissues (Freire et al., 2008; Henry et al., 2012; Lignot and Charmantier, 2015).

While osmoregulation in adult crustaceans has been widely studied, our knowledge of how osmoregulatory ability, and the structures that provide that function, develop during ontogeny, is not well documented (Anger, 2003; Charmantier, 1998; Charmantier and Charmantier-Daures, 2001; Lignot and Charmantier, 2015).

The ontogeny of whole animal osmo-and iono-regulation has been investigated mainly in crustaceans that undergo indirect development, such as crabs (Augusto et al., 2009; Brown and Terwilliger, 2007; Charmantier and Charmantier-Daures, 1994), shrimps (Bouaricha et al., 1994; Cieluch et al., 2005; Felder et al., 1986), crayfish (Susanto and Charmantier, 2002) and lobsters (Charmantier and Aiken, 1987; Dall, 1970). Some attention has also been paid to the structures, and biochemical mechanisms, associated with developing osmoregulatory ability, such as the ephemeral crustacean larval salt gland (Conte, 1984), and the onset of Na^+-K^+-ATPase and carbonic anhydrase activity (Lignot and Charmantier, 2015). This work lends support to the incomplete adult hypothesis, where improvement in osmoregulatory capacity appears to coincide with a major metamorphosis. Such support is more equivocal in direct developing groups such as the peracarids: mysids, isopods and amphipods. Peracarids lay and brood their eggs in a marsupium, a modification of the ventral groove, within which semi- and fully terrestrial species, such as the amphipods (Morritt and Spicer, 1996a) and isopods (Hornung, 2011) can regulate the osmotic concentration of the exosomatic water

those eggs are immersed in. Even though the osmotic concentration of the marsupial fluid of the semi-terrestrial amphipod *Orchestia gammarellus* is tightly regulated, all embryonic stages display a strong hyper-hypo-regulation (Morritt and Spicer, 1999). Interestingly, the regulation is much weaker in immediately post hatch individuals (Morritt and Spicer, 1999). In this species, new hatchlings are retained within the marsupium for a further 10 days until they (re)develop the adult pattern of osmoregulation (Morritt and Spicer, 1996b). This does not support the incomplete adult hypothesis.

There is some evidence for maternal control of osmolality in the marsupium of aquatic peracarid crustaceans but only for two species, the mysid *Praunus flexuosus* (Mclusky and Heard, 1971) and the isopod *Sphaeroma serratum* (Charmantier and Charmantier-Daures, 1994). There is no published information for amphipods, although preliminary experiments investigating the osmolality of water within the marsupium of *Marinogammarus marinus* and *Gammarus chevreuxi* kept in different salinities, point to no maternal control of marsupium water osmolality. This is perhaps not surprising given the more open nature of the gammarid marsupium, and the fact that the pleopods beating in the posterior part of the marsupium ensure a constant replenishment of groove water (Dahl, 1978). Therefore, it is unlikely that there is effective maternal osmotic control in aquatic amphipods, similar to that found in more semi- and fully terrestrial species.

Our knowledge of embryonic osmoregulation in fully aquatic isopods and amphipods is limited to one species of each. Embryos of the euryhaline isopod *Sphaeroma serratum* displayed a weak osmoregulatory ability throughout development but began to improve early after hatching (Charmantier and Charmantier-Daures, 1994). The ontogeny of osmoregulation in the euryhaline brackishwater amphipod

Gammarus duebeni is more complex than that of the semi-terrestrial amphipod *O. gammarellus*, and the aquatic isopod *S. serratum* (Morritt and Spicer, 1995). Early embryos of *G. duebeni* (Stage 2, characterised by a prominent dorsal organ) displayed a hyper-iso-osmotic pattern of regulation of perivitelline fluid when exposed to a range of environmental salinities. This regulation was present before the appearance of the coxal gills possibly *via* extra-embryonic structures, namely the vitelline membrane and/or the dorsal organ (DO) (Morritt and Spicer, 1995). This pattern was also observed for the haemolymph of new hatchlings but, interestingly, stage 5-7 embryos (pre-hatch but undergoing marked organogenesis) displayed a transient hyper-hypo-osmotic pattern of regulation, similar to the embryonic pattern in the semi-terrestrial *O. gammarellus*. This development and loss of what might be regarded as the most complex form of osmoregulation (i.e. hyper-hypo-regulation), does not support the incomplete adult hypothesis. Clearly, there are differences in the patterns and ontogeny of osmoregulation between euryhaline embryonic isopods and amphipods, and differences between semi-terrestrial and aquatic amphipods, but current knowledge does not allow for generalisations. Furthermore, there is little information on the structures, and no information on the molecular basis, responsible for these regulations or their ontogenies.

Therefore, the aim of this study was to test the incomplete adult hypothesis of Adolph, (1968) and Spicer and Gaston (1999) by 1) characterising the ontogeny of osmoregulation of a congeneric euryhaline amphipod, at different levels of biological organisation, and 2) compare and contrast the picture that emerges specifically with *G. duebeni* and *O. gammarellus*, the only other amphipod species for which we have comparable information. Consequently, we investigated the ontogeny of the structure

and function of putative osmoregulatory tissues, together with the regulation of key osmoregulatory genes, in the brackishwater amphipod *Gammarus chevreuxi*. We predicted that, like in *G. duebeni,* the most complex pattern of regulation (hyper-hypo-) would appear early in embryonic development and then revert to the less complex hyper-iso- regulation before or around hatching. *Gammarus chevreuxi* was chosen as it is a euryhaline species, exposed to freshwater (salinity $\approx$ 0) and full strength sea water (salinity >30) within a tidal cycle, its embryonic development is well characterised, it is lab-hardy (Sexton, 1928), and its transcriptome has recently been sequenced (Collins et al., 2017; Truebano et al., 2013).

2. Materials and Methods

1.1. Collection and husbandry of animals

Amphipods were collected during low tide using a kick net (mesh size = 500 µm) from the Plym estuary, Devon (50° 23' 24'' N, 4° 5' 7'' W). A Star:Oddi DST CTD logger was deployed for 48 h at the collection site to measure tidal salinity variation. This population experiences a salinity pulse, when salinities reach values of above 30 for approximately 4 h during the tidal cycle. Amphipods were returned to a temperature-controlled laboratory and sorted into stock aquaria containing diluted sea water (vol. = 25 L, T =15 °C, S = 15±1, light = 12h:12h L:D cycle). Amphipods were held in the stock aquaria in pre-exposure conditions for a minimum of two weeks, and fed carrot *ad libitum*. Water changes were performed weekly. After four weeks, pre-copula pairs were isolated from the stock populations and transferred to individual aquaria (vol. = 0.5 L) maintained under the same conditions as the stock aquaria. Males were removed immediately once the pairs had separated and eggs

were visible in the marsupium of the female. Individual eggs of different developmental stages, hatchlings or adults were removed as required to supply the experiments described below.

1.2. Ontogeny of putative osmoregulatory organs

1.2.1. Morphological observations

Each day after fertilization, eggs from a proportion of the females were brushed out of the marsupium using a fine paint brush as described by (Morritt and Spicer, 1995), and placed in double filtered autoclaved sea water adjusted to the same conditions as those used for the pre-exposure period. Eggs were observed and photographed under a high powered light microscope coupled to an Allied Vision Pike 210C real time digital camera (Allied Vision Technology, Germany) and the timing of the appearance and development of putative osmoregulatory structures recorded.

1.2.2. Scanning electron microscopy (SEM) of embryos

To further characterise the structure and position of the DO, embryos (>14 dpf) were examined under SEM. Eggs were fixed in 2.5 % glutaraldehyde in diluted sea water for 12 h and rinsed twice for 15 min in sodium cacodylate buffer (0.1 M, pH 7.2) at 4 ˚ C. Eggs were then placed in 30 % ethanol, and carefully dechorionated using two fine dissecting needles. They were then further dehydrated through a graded ethanol series ranging from 30 % through 50, 70, 90 and 100 % and critically point dried in an Emitech K850 critical point drier (Quorum Technologies Ltd., UK). Fully dried

149 samples were coated with gold and examined using a JEOL JSM 5600 LV scanning
150 electron microscope (Jeol, Japan).

151

152 1.2.3. Staining of ion regulatory tissue and permeable areas in embryos

153 Silver staining was used to identify areas of active ion uptake on embryos. Attempts
154 to stain embryos at stages before the DO is visible (< 6 dpf) resulted in cell lysis.
155 Accordingly, the location of putative ion regulatory tissues and permeable areas was
156 determined for eggs 6, 9, 14 and 18 dpf. These stages were selected based on initial
157 morphological observations and mark the appearance and development of structures
158 with putative osmoregulatory function (i.e. DO and gills). Immediately upon removal
159 from the brood pouch, eggs were briefly rinsed twice in deionised water, transferred
160 to a 5 g L^{-1} $AgNO_3$ solution for 5-7 min and washed again for 10 min in deionised
161 water. Observations of staining were made under a high powered light microscope
162 and digital images obtained as described above.

163

164 *1.3.* Ontogeny of osmo- and iono-regulation

165

166 1.3.1. Osmoregulatory patterns in embryos

167 The osmolality of homogenised eggs was measured at different stages of
168 development exposed to three salinity treatments (S= 2, 15 and 33) for 24 h. This
169 exposure time was shorter than used for the adults primarily to ensure the exposures
170 of quite discrete and different periods in embryonic development. Although we have
171 no information for embryonic amphipods, we know that salinity acclimation in adults
172 can be very rapid, with most of the change occurring during the first few hours of

transfer, and new steady states achieved well within 48 h (Bolt, 1983; Dorgelo, 1974).

Samples were selected at stages representing embryos in which the DO had not started to develop (<48 hpf), the DO was present but not the gills (9 dpf), and both the DO and gills were present (14 -18 dpf). For each salinity treatment and stage, batches of eggs were carefully removed from the brood pouch and photographed as previously described. The length and width of each egg was measured using ImageJ (Schneider et al., 2012) and used to calculate egg volume as an oblate ellipsoid. Pools of eggs (n = 20 per salinity) were washed twice in milliQ water, and homogenised with a manual homogeniser. The osmolality of the homogenates (0.03 – 0.05 µL) was determined using a direct-reading nanolitre osmometer (Clifton Technical Physics) (Morritt and Spicer, 1996). Variation between replicates was < 35 mOsm Kg^{-1}. Homogenates, rather than haemolymph or perivitelline fluid, have been used previously to investigate the ontogeny of osmoregulation in crab embryos (Seneviratna, 2006).

1.3.2. Osmo- and iono- regulatory patterns in adults

Analysis of haemolymph osmolality and key ionic concentrations (sodium (Na^+), calcium (Ca^{2+}) and magnesium (Mg^{2+})) was performed for large adult *G. chevreuxi* (>10 mm length) exposed to one of four salinity treatments (S = 2, 10, 25 and 35) for seven days. Haemolymph was extracted by inserting the needle of a microsyringe (vol.= 10 µL) directly into the heart, dorsally through the second and third dorsal plates of the pereon. The osmolality of untreated pooled haemolymph samples (n = 12-15 individuals) was measured in duplicate using a Vapro 5520 vapour pressure osmometer (Wescor, USA) fitted with a reduced volume sample holder (2 µL). as

198 described above. The ionic content of the haemolymph was estimated as follows:
199 Immediately after extraction, 0.5 µL haemolymph (3-5 individuals) was pooled in a
200 microcentrifuge tube, diluted in 1 mL milliQ water, and refrigerated before analysis.
201 Ionic concentrations in adult haemolymph and corresponding treatment media were
202 measured using inductively coupled plasma optical emission spectroscopy (ICP-
203 OES). Samples were analysed for Na^+, Mg^{2+} and Ca^{2+} ions, using a Varian 725ES
204 ICP-OES instrument (Varian, Australia) fitted with a V-groove nebuliser coupled with
205 a Sturman-Masters spray chamber, calibrated using four standards and one blank.
206 Operating parameters were set to a forward power of 1.4 kW, plasma flow of 15 L
207 min^{-1}, auxiliary flow of 1.5 L min^{-1} and nebuliser gas flow of 0.68 L min^{-1}, a viewing
208 height of 8 mm above the load coil and a read time of 4 s.

209

210 1.3.3. Gene expression analysis of Na^+/K^+-ATPase

211 The transcriptome of *G. chevreuxi* at different embryonic stages has been recently
212 sequenced, assembled and annotated as described in Truebano et al (2016). Six
213 transcripts were putatively identified as Na^+/K^+-ATPases beta (four transcripts) or
214 alpha (two transcripts) subunits. Of these, we found differences in expression
215 between early and late developmental stages in two mRNA transcripts putatively
216 identified as Na^+/K^+-ATPase alpha subunit (**GeneBank accession no.**
217 **HADC01011431.1 and HADC01011432.1,** length: 4398 and 4317 bp respectively).
218 To investigate whether the two transcripts are differentially expressed during
219 ontogeny across the developmental stages studied here, expression analysis was
220 carried out in eggs at different developmental stages using qPCR. Expression
221 patterns of both transcripts were investigated in pools of embryos pre-DO (<16 cells,
222 <48 hpf), DO present (9 dpf), gills present (18 dpf) and adults. The <16-cell embryos

were included in order to determine whether there is evidence of iono-regulatory capacity in the earliest stages of development. Total RNA was isolated from three pools of 50 embryos per each of the four developmental stages (n=12) or three pools of 10 adults using the Reliaprep RNA tissue Miniprep System (Promega, Southampton, UK) following manufacturer's instructions. RNA purity and concentrations were measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Loughborough, UK) and integrity was assessed using gel electrophoresis. 200 ng total RNA was reversed transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, California, USA). Samples were amplified in triplicate in 10 µL reactions containing 2 µL cDNA (1:10 dilution) in the presence of SYBR Green (iTaq Universal SYBR Green Supermix, BioRad, Hertfordshire, UK) in a StepOne Real-Time PCR system (Life Technologies, Paisley, UK) according to manufacturers' instructions. A melt curve was added to each run. Ct values were normalised to the geometric mean of 18S ribosomal subunit (18S) and elongation factor (EFα) after checking their stability via geNorm (Vandesompele, et al., 2002). Data are presented as ΔCt ($Ct_{reference}$-Ct_{target}). Fold changes were calculated we $2^{\Delta Ct}$. Primers were designed by qStandards (EFα and 18S, Edgware, UK) or Primerdesign Ltd ($Na^{+/}K^{+}$-ATPase, Southampton, UK) (Table 1).

Table 1. Primer information for two target Na^{+}/K^{+}-ATPase transcripts (named long and short for convenience) and the reference genes elongation factor (EFα) and 18S ribosomal subunit (18S).

Target gene	Primer Sequence	Amplicon length (bp)	Tm (°C)	Efficiency (%)	R^2
Na^{+}/K^{+}-ATPase long	F: gccacaaaaatgagtgatagcg R: tctcccttgaaagtacggttatc	92	75.3	90-100	-

Na^+/K^+-ATPase short	F: tttactgataataccttggatactgtg R: ttcgccttcttcttcgaatcac	97	70.3	90-100	-
EFα	F: caaccgtctgtacatgaaggct R: accgaaggtccagatcttcatgg	163	87.3	103	0.999
18S	F: tgaacgaaagttagaggatcgaagg R: cggattgatggttggcatcgt	77	80.7	98	0.999

2. Results

2.1. Ontogeny of putative osmoregulatory organs

2.1.1. Morphological observations

Embryogenesis (fertilization to hatching) under the experimental conditions (T = 15°C, S = 15) took 400-450 h in *Gammarus chevreuxi* (Figure 1).

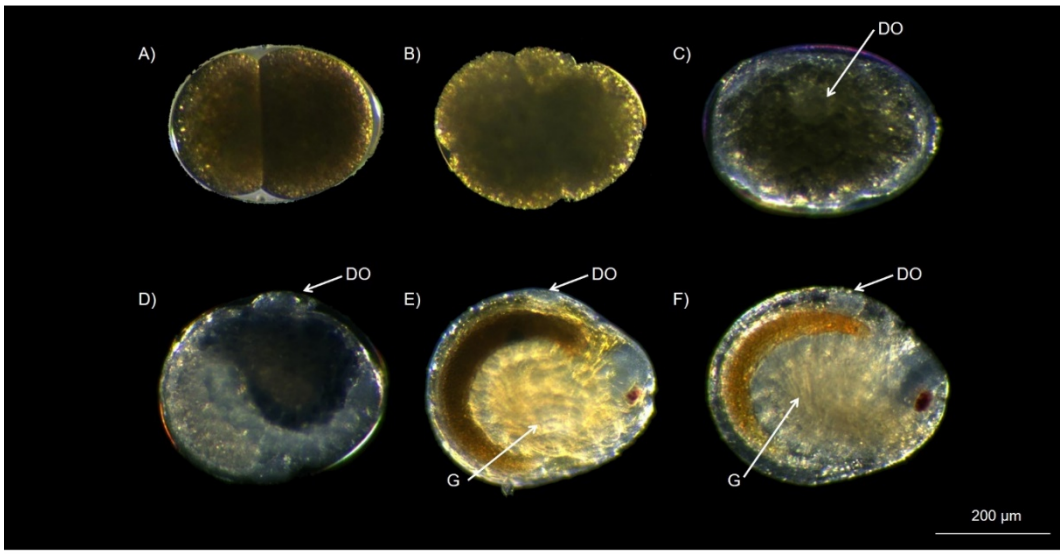


Figure 1. Light microscopy images of *Gammarus chevreuxi* embryos developed in the marsupium of females and removed for examination at A) 2-cells, B) <16 cells,

C) 6 dpf, D) 9 dpf, E) 14 dpf and F) 18 dpf. DO and G indicate embryonic dorsal organ and gills respectively. Scale bar = 200 µm.

The first cell division (2-cell stage) occurred within 10 hpf (Figure 1A), fertilization being defined as the time at which the mating pair separates. Cell boundaries are clearly visible under light microscopy until approximately the 16-cell embryo (Figure 1B), after which time individual cells are not easily identifiable. The timing of the initial aggregation of cells forming the DO cannot be identified visually using light microscopy during early development. However, 5-6 days after fertilization, the DO is easily identifiable unaided under light microscopy as an aggregation of cells located dorsally (e.g. 6 dpf, Figure 1C). It becomes a well-defined structure that remains located on the anterior dorsal surface through development (Figure 1D, E, F). Upon fixing and dechorionation of the embryos, the DO appears the only point of attachment between the egg membrane and the embryo.

2.1.2. Scanning electron microscopy (SEM) of embryos

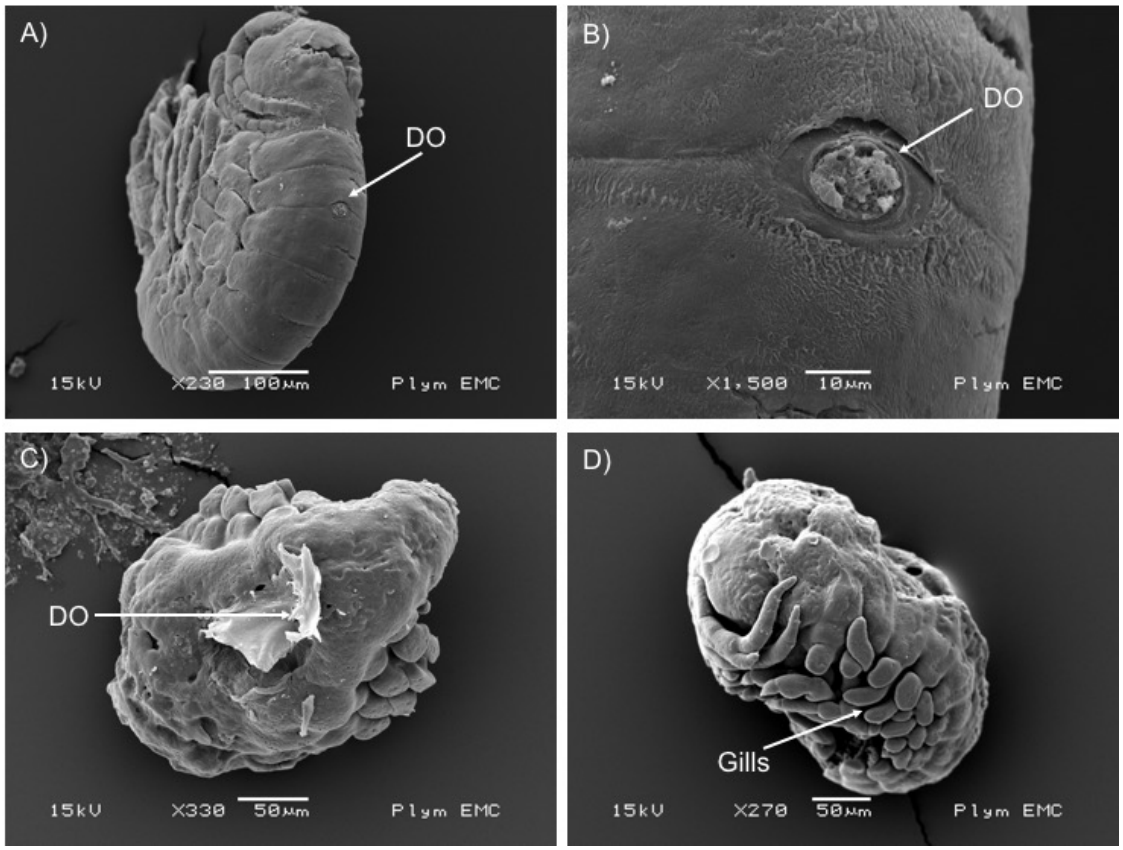


Figure 2. Scanning electron microscopy images of dechorionated *Gammarus chevreuxi* embryos fixed at 14 dpf indicating, A) the position of the embryonic dorsal organ, B) the dorsal organ, C) the association between the dorsal organ and the chorion, and D) the position of the gills. DO and G indicate embryonic dorsal organ and gills respectively. Scale bars (10-100 µm) and magnifications (x230-1500) are shown for each image.

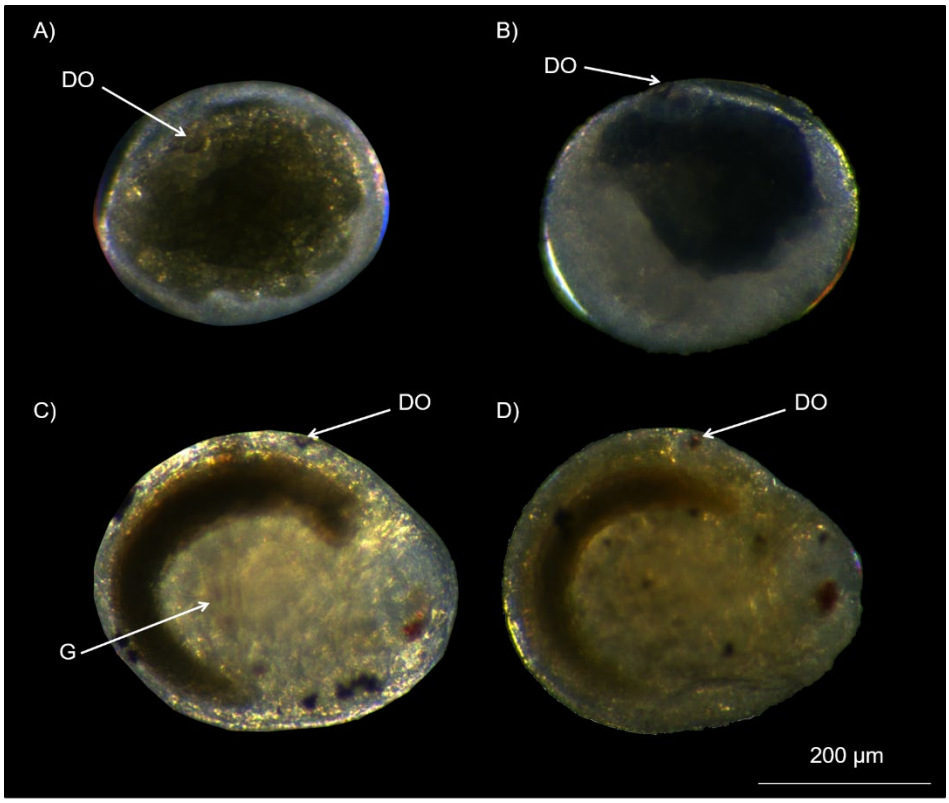
SEM of 14 dpf individuals clearly shows the DO as an oval structure (approx. 15 µm diameter), located on the dorsal surface of the embryo between the second and third cuticular segments of the pereon (Figure 2A, B). Figure 2C shows a fragment of the egg membrane apparently attached to the area of the DO, providing further evidence that this is the only point of attachment between the embryo and the egg membrane. The gills are also observable in the same individual (Figure 2D), confirming the co-

289 occurrence of both organs in the late stages of development. As the gills are located
290 beneath the coxal plates, their development is difficult to visualise in live embryos.
291 Therefore, it is possible that rudimentary gills develop earlier than suggested here.
292 The gills are clearly visible under light microscopy at the late stages (>12 dpf, Figure
293 2F, G).

294

295 2.1.3. Staining of ion regulatory tissue and permeable areas in embryos

296



297

298 Figure 3. Light microscopy images of *Gammarus chevreuxi* embryos developed in
299 the female marsupium. Embryos were stained with $AgNO_3$ at A) 6 dpf, B) 9 dpf, C)
300 14 dpf, and D) 18 dpf. Arrows with accompanying letter indicate silver stained areas,
301 corresponding with the locations of the embryonic dorsal organ (DO) and gills (G).
302 Scale bar = 200µm.

Silver-stained embryos at 6, 9, 14 and 18 dpf present an oval shaped dark area with a darker outer ring that can be identified on the surface of the embryo (Figure 3), corresponding with the position of the DO, as shown in Figure 2. The stained area is likely to be a silver precipitate, which is indicative of active ion uptake. At 14 dpf, weak staining of the gills is observed. Note that the lower intensity does not necessarily indicate a lower active uptake of ions, but could equally indicate a decrease in permeability.

2.2. Ontogeny of osmo- and iono-regulation

2.2.1. Osmoregulatory patterns in embryos

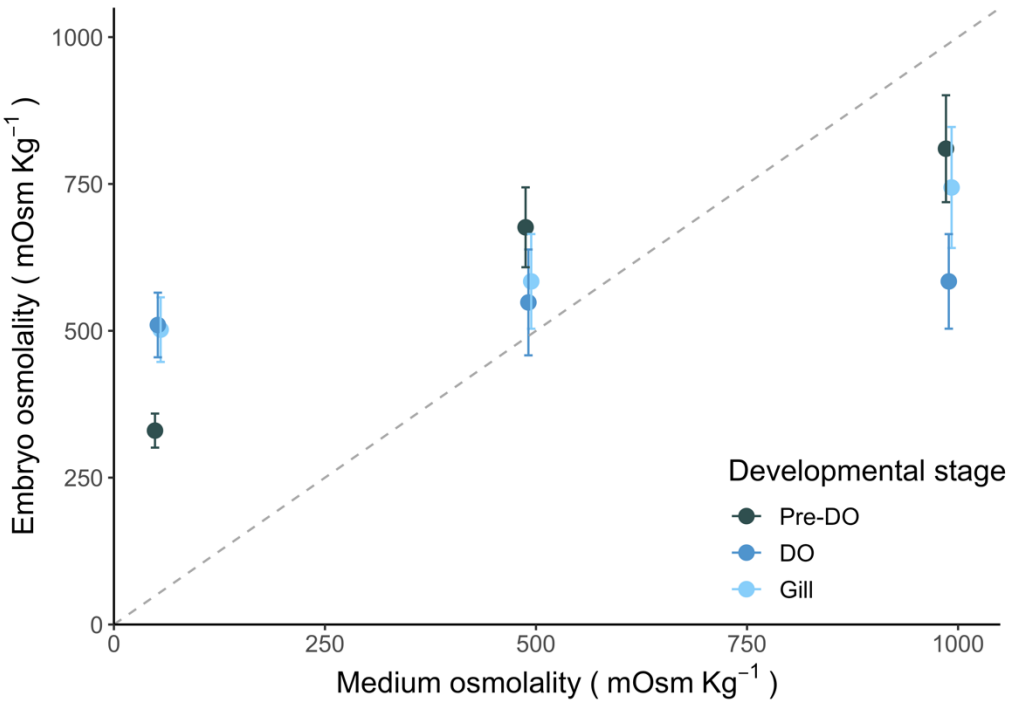
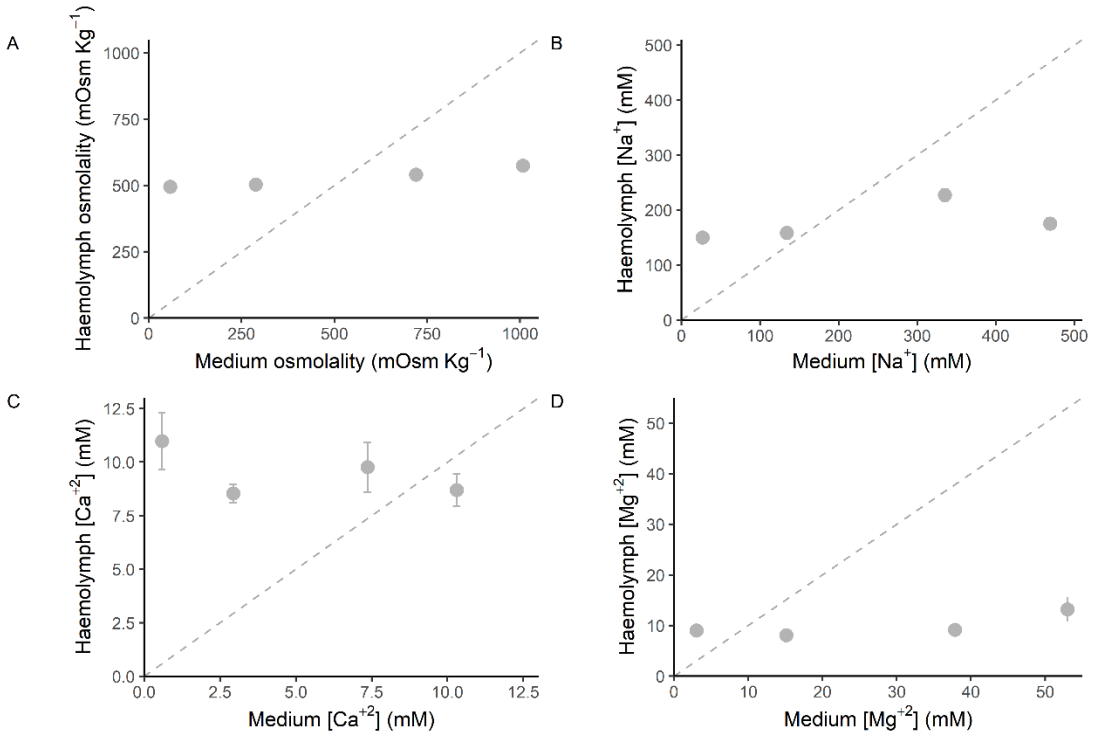


Figure 4. Osmolality of *Gammarus chevreuxi* homogenate of embryos removed from the mother at three different stages of development (i.e. pre-dorsal organ (pre-DO, dark grey), dorsal organ present (DO, dark blue), and gills present (Gill, light blue)) and subsequently exposed to salinities of 2, 15 and 33 for 24 h *in vitro*. Points represent mean concentrations and standard deviations of three biological replicates per treatment, each consisting of pools of 25 embryos. Broken line represents the isosmotic line.

For *G. chevreuxi* embryos, extraction of periembryonic fluid was not possible until approximately 8 dpf. Therefore, in order to compare osmoregulatory capacities of embryos before and after the development of the DO and gills, osmoregulatory curves were produced using osmolality of embryo homogenates, from embryos removed from the marsupium and exposed to different salinities *in vitro* (S= 2, 15 and 33). The osmolality of the homogenate is expressed as a function of the corresponding external osmolality (Figure 4). All embryonic stages investigated showed some degree of hyper-hypo-osmoregulation. At a salinity of 35, eggs were hyposmotic to the external medium (989 mOsm Kg^{-1}) by several hundred mOsm Kg^{-1} in the DO and gill stages (mean±SD= 584±80.5 and 744±103 mOsm Kg^{-1} respectively) (Figure 4 B,C), with less of a difference with the medium before DO development (mean±SD= 810±91 mOsm Kg^{-1}) (Figure 4A). At a salinity of 2, eggs from all stages were hyperosmotic to the external medium (52 mOsm Kg^{-1}), with the pre-DO stage being closest to the isosmotic line (mean±SD= 330±29, 510±55 and 502±55 mOsm Kg^{-1} for pre-DO, DO and gill respectively). Differences between the stages diminished at salinity 15.

343 2.2.2. Osmo- and iono-regulatory patterns in adults

344



345

346

347 Figure 5. Osmotic and ionic regulation in adult *Gammarus chevreuxi* acclimated to
348 four salinities (S= 2, 10, 25 and 35) for seven days. Haemolymph (A) osmolality (B)
349 sodium (Na^{+}) concentration, (C) calcium (Ca^{2+}) concentration and (D) magnesium
350 (Mg^{2+}) concentration. Points represent mean concentrations and standard errors of
351 pooled biological replicates from the respective treatments (n= 12, 15, 15 and 15 for
352 salinities 2, 10, 25 and 35 respectively (A); n= 40, 44, 40 and 19 for salinities of 2,
353 10, 25 and 35 respectively (B-D)). Broken lines represent isosmotic lines.

354

355 Regulation of osmolality and selected key ions in the haemolymph of amphipods
356 exposed to different environmental salinities is presented in Figure 5. In each case, a

hyper-hypo-regulation pattern was observed. Total osmolality and the concentrations of all three ions were tightly regulated across the salinities tested.

2.2.3. Gene expression profiles of putative osmoregulatory genes during ontogeny

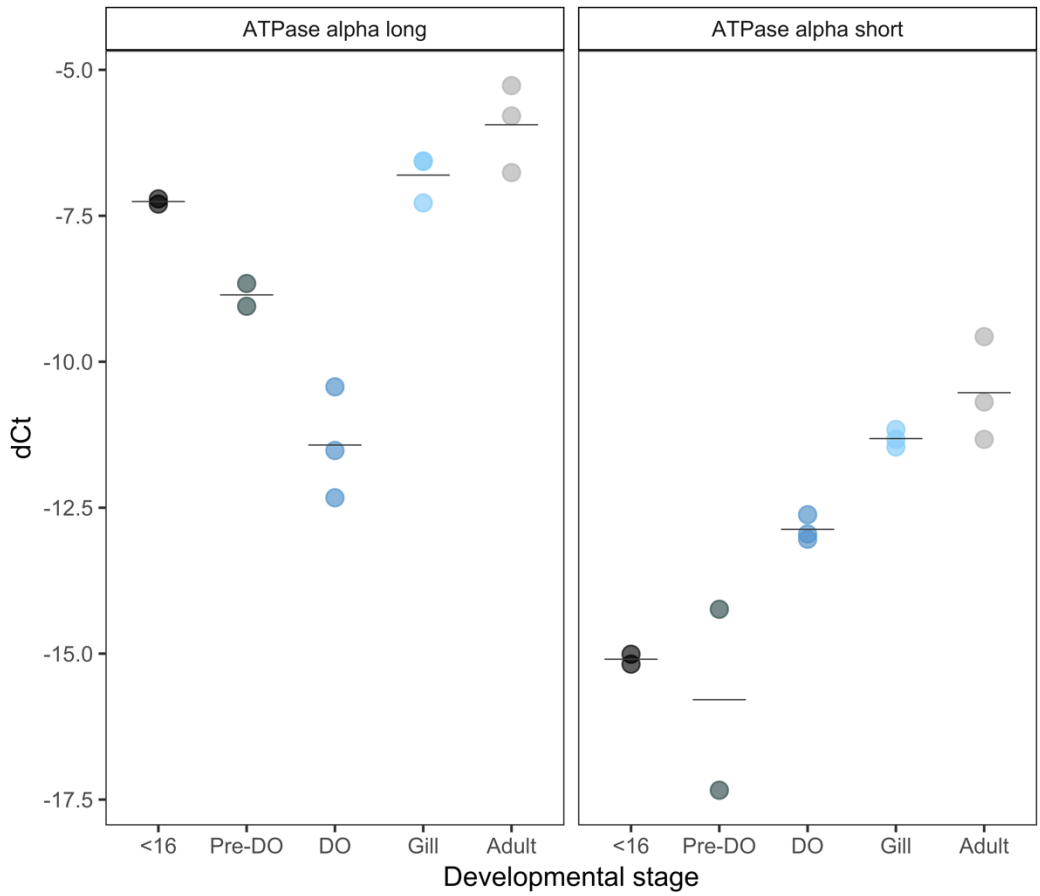


Figure 6. Expression levels (dCt) for two isoforms putatively identified as Na^+/K^+-ATPase alpha subunit in four embryonic stages corresponding with <16-cells (<24 hpf), pre-dorsal organ (pre-DO, <48 h), dorsal organ present (DO, 9 dpf), gills present (Gill, 18 dpf) and adults (*n* = 2-3 pools of 50 embryos per developmental stage, and n= 3 pools of 10 adults). Transcripts are labelled ATPase alpha long (transcript length= 4398 bp) and ATPase alpha short (transcript length= 4317 bp) for convenience.

Both Na^+/K^+-ATPase transcripts were expressed throughout development. The expression of the longer transcript differed significantly between stages (ANOVA, $F_{4,8}$=32.39, P<0.001), showing greater expression at <16 cells, followed by a significant downregulation in dorsal organ embryos (approximately 30% downregulation compared to the <16-cell stage) before increasing again in embryos presenting gills and in adults to levels similar to those of the <16-cell embryos (1.37 and 2.49-fold increase respectively compared to <16-cell embryos). The expression of the short transcript increased with ontogeny (ANOVA, $F_{4,8}$=15.41, P<0.001) being lowest in the early stages (<16 cells and pre-DO) and highest at the late stages following a 4.67, 13.68 and 23.59-fold increase in dorsal organ embryos, gill embryos and adults respectively in relation to the <16-cell stage (Figure 6). The expression ratio between the two transcripts was reasonably stable throughout ontogeny with the long transcript having higher expression levels (ΔCt long - ΔCt short= 1.66-2.08, except for dorsal organ embryos, where expression levels were most similar 1.13).

3. Discussion

3.1. Adult osmo- and iono-regulatory pattern

Adult *Gammarus chevreuxi* exhibited a pronounced hyper-hypo-osmoregulatory pattern. The only other gammarideans for which this pattern is recorded is the high shore *Orchestia gammarellus* (Moore and Francis, 1985; Morritt, 1988) and individuals of a brackishwater population of the predominantly marine amphipod

Gammarus oceanicus (Normant et al., 2005). However, in *G. oceanicus* where population differentiation is well attested (Croker and Gable, 1977), individuals from more oceanic populations show a hyper-iso- osmoregulatory pattern (Aarset and Zachariassen, 1988; Brodie and Halcrow, 1978) in common with the adult stage of many marine or estuarine gammaridean amphipod species (Dorgelo, 1974; Kiko et al., 2009; Werntz, 1963). The strong osmoregulatory capacity of adult *G. chevreuxi*, is matched by an equally strong pattern of haemolymph ion regulation including sodium, which is a major component of effective osmoregulation (Lignot and Charmantier, 2015).

3.2. Ontogeny of osmoregulation and the dorsal organ

The adult pattern of hyper-hypo-osmoregulation appeared in embryonic *G. chevreuxi* well before the ontogeny of the gills (14 dpf), the presumed primary site of ion exchange in crustaceans (Freire et al., 2008; Henry et al., 2012), and the pattern did not change thereafter. This points to an osmoregulatory role for extrabranchial structures prior to gill ontogeny. Appearing at 6 dpf the oval shaped, silver-stained area on the egg surface, which has a darker outer ring surrounding it, corresponds with the position of the DO. This is consistent with the DO ultrastructural description of Meschenmoser (1989). The most dense staining occurs where the DO connects to the vitelline membrane in *G. chevreuxi* (this study) and other peracarids (Bregazzi, 1973; Meschenmoser, 1989), and is likely to be indicative of active ion pumping. While the DO may well be implicated in osmoregulation, the adult pattern of hyper-hypo-regulation in *G. chevreuxi* was present before the DO becomes visible (6dpf). However, before 6dpf, both hyper- and hypo-regulatory ability were not as strong as they were post-DO formation. The results of silver staining and the correlation

between improved osmoregulation and the appearance of the DO, support the idea that, for *G. chevreuxi*, and in many other amphipod and isopod crustaceans, the DO is involved in osmotic and ionic regulation (Bregazzi, 1973; Martin and Laverack, 1992; Meschenmoser, 1989; Morritt and Spicer, 1995; Strömberg, 1972; Vlasblom and Bolier, 1971; Wright and O'Donnell, 2010).

At 14 dpf, the DO began to degenerate in *G. chevreuxi* and at the same time the gills became visible and showed weak silver staining. The centre of the DO stained (unlike the vitelline membrane) in all developmental stages, up until the point where it had totally degenerated, suggesting it remained functional until very late in embryonic development. Interestingly, like *G. chevreuxi,* the coxal gills of late stage *G. duebeni* embryos also showed little stain retention. Morritt and Spicer (1995) suggested therefore, that the coxal gills may only provide major osmoregulatory function post-hatching in this species, as it is only then the gills stain darkly. However, the lower staining intensity could equally be indicative of a decrease in chorion permeability.

3.3. Gene expression and osmoregulation

Na^+/K^+-ATPases are ubiquitous, highly conserved transport proteins consisting of alpha and beta subunits. The alpha subunit is the catalytic unit, and the beta subunit facilitates insertion in the membrane (Skou, 1990). In adult crustaceans, Na^+/K^+-ATPase activity is largely responsible for epithelial movement of monovalent ions across specialized ion-regulatory cells and tissues, mostly located in the gills (Leone et al., 2017; Lignot and Charmantier, 2015). Its activity is associated with osmoregulation in euryhaline crustaceans, with significant increases in activity

444 demonstrable during both hyper- and hypo-osmoregulation (reviewed by Lucu and
445 Towle, 2003). Long-term increases in activity are likely a result of enzyme activation
446 and *de novo* protein synthesis *via* enhanced transcription (Havird et al., 2013). An
447 increase in the activity of Na^+/K^+ -ATPase during embryonic development has been
448 recorded in a wide range of decapod species (Felder et al., 1986; Ituarte et al., 2008;
449 Taylor and Seneviratna, 2005; Wilder et al., 2001). While gill Na^+/K^+ -ATPase activity
450 has been measured in adult amphipods (Brooks and Lloyd Mills, 2006), nothing is
451 known of how enzymatic activity, or its associated gene expression, changes during
452 embryonic development in these peracarids.

453

454 In *G. chevreuxi*, we found evidence of regulation during ontogeny of two different
455 transcripts encoding the alpha subunit. More than one copy of the Na^+/K^+ ATPase
456 alpha gene is reported in crustaceans including the waterflea *Daphnia pulex* (Macias
457 et al., 1991), the brine shrimp *Artemia franciscana* (Baxter-Lowe et al., 1989) and the
458 barnacle *Balanus improvisus* (Lind et al., 2013), which is divided into two classes;
459 Na^+/K^+-ATPase 1 and 2. In addition, some crustacean species present different
460 isoforms of the alpha 1 variant, representing different splice variants. This has been
461 well characterised in the barnacle, *B. improvisus,* where the long and short forms
462 differ by 27 amino acids at the N-terminus. Analysis of the transcripts identified in *G.*
463 *chevreuxi*, suggests that these mRNAs represent a long and short slice variant
464 differing only by 81 bp. Alignment of these mRNA sequences to the long and short
465 Na^+/K^+ ATPase alpha 1 splice variants identified in the barnacle *B. improvisus*, the
466 shrimp *Penaeus monodon* and the crab *Pachygrapus marmoratus* (alignment given
467 in Lind et al., (2013)), revealed similarity between the two main variants for each
468 individual including or excluding the 27 amino acids. This suggests that the *G.*

469 *chevreuxi* transcripts identified herein belong to the Na^+/K^+ ATPase alpha 1 class,
470 and are homologous products of alternative splicing of a 27 amino acid exon (Figure
471 S1). While the functional importance of this exon in the encoded proteins is not
472 known, we have observed differences in expression patterns between the two
473 homologs. Expression of both transcripts was detected in all three developmental
474 stages examined. This supports the idea that ion transporting activity may occur
475 before the formation of osmoregulatory organs, potentially through Na^+/K^+-ATPase-
476 rich ionocytes. Such activity may be required to ensure the maintenance of osmotic
477 balance during hydration. Overall, the expression of both transcripts was greatest in
478 the late stages, embryos with gills and adults, which is consistent with the increased
479 activity levels observed during ontogeny in other crustaceans (Felder et al., 1986;
480 Ituarte et al., 2008; Taylor and Seneviratna, 2005; Wilder et al., 2001). However,
481 while the expression in one of the transcripts increased with ontogeny, the other
482 showed complex patterns, with reduced expression after the appearance of the
483 dorsal organ, compared to all other stages, suggesting different stage specific splice
484 variants may play a role during ontogeny. Differences in expression of different
485 splice variants of the alpha subunit between life stages have been described in the
486 barnacle *Amphibalanus* (as *Balanus) improvisus*, with dominance of a longer mRNA
487 over the short variant in cyprids, whereas in the adult, the short isoform was clearly
488 dominant (Lind et al., 2013). Interestingly, the long mRNA form is up-regulated in
489 relation to the short form during low salinity conditions, indicating the long protein
490 might have a more prominent functional role in maintaining haemolymph
491 hyperosmotic to the surrounding water under low salinity (Lind et al., 2013). The
492 expression ratio between the two transcripts remain constant throughout ontogeny,
493 except in dorsal organ embryos, where expression of both transcripts was most

494 similar. Differences in the ratio between two different isoforms during development
495 have been previously observed in the brine shrimp *Artemia salina* (Salon et al.,
496 1989), but the functional significance of these changes is not known and warrants
497 further investigation. It should be acknowledged, that, while the ontogenic changes in
498 expression of these two transcripts is interesting, they only represent a minor
499 component of the osmoregulatory genes in *G. chevreuxi.* Further gene expression
500 profiling across developmental stages and salinities is required to fully characterise
501 the molecular mechanisms underpinning the development of osmoregulation in this
502 species.

503

504 3.4. Comparison with other amphipod species

505 The ontogeny of osmoregulation in the three gammaridean species that have been
506 investigated to date all fit “Pattern 2” of Charmantier and Charmantier-Daures
507 (2001). Here, the ‘adult’ pattern is established around hatching, and adults are
508 euryhaline and can live in environments where salinity is high, low or variable.

509 While the ontogeny and development of osmoregulatory function in embryonic *G.*
510 *chevreuxi* was similar to that of embryonic *O. gammarellus* (Morritt and Spicer,
511 1999, 1996c), it differed from embryonic *G. duebeni* (Morritt and Spicer, 1995). A
512 hyper-hypo-regulatory pattern was already established in the earliest embryonic
513 (stage 2/3) examined of *O. gammarellus* and persisted through to hatching, although
514 it disappeared and developed again in hatchlings (Morritt and Spicer, 1999).
515 Paradoxically, the embryonic pattern was stronger than that of the adult (Morritt and
516 Spicer, 1996b) even though embryos are retained within the brood pouch where
517 there is tight maternal control of osmotic pressure of the exosomatic water within

518 (Morritt and Spicer, 1996a). This species can be subject to salinity extremes over a
519 number of different timescales (Moore and Francis, 1985).

520

521 *G. duebeni,* although more aquatic than *O. gammarellus*, also lives in habitats
522 subjected to large salinity variations but shows a slightly different osmoregulatory
523 pattern. Adult and early embryonic (stage 2/3) *G. duebeni* are hyper-iso-
524 osmoregulators. However, in medium and late embryos (stages 5-7) regulation was
525 hyper-hypo-osmotic, before reverting to hyper-isosmotic in hatchlings (Morritt and
526 Spicer, 1995). The significance of this transient ability to hyper-hypo-regulate is not
527 known, although Morritt and Spicer (1995) suggested it may be associated with the
528 appearance of the coxal gills, the putative primary osmoregulatory organs in
529 juveniles and adults, and with the concomitant disappearance of the DO. However,
530 while in *G. duebeni* the DO begins to degenerate around stage 5 and has
531 disappeared by hatching, in *G. chevreuxi* the DO remains visible right up until
532 hatching.

533 To conclude, the ontogeny of osmoregulatory function in the gammaridean
534 amphipod *G. chevreuxi* is consistent with the incomplete adult hypothesis proposed
535 by Adolph (1968) and Spicer and Gaston (1999), However the ontogenies of that
536 same function in the closely-related *G. duebeni* and in the talitrid amphipod
537 *Orchestia gammarellus* are more consistent with the physiological competency
538 hypothesis.

539

540

541 **Figure captions**

Figure 1. Light microscopy images of *Gammarus chevreuxi* embryos developed in the marsupium of females and removed for examination at A) 2-cells, B) <16 cells, C) 6 dpf, D) 9 dpf, E) 14 dpf and F) 18 dpf. DO and G indicate embryonic dorsal organ and gills respectively. Scale bar = 200 µm.

Figure 2. Scanning electron microscopy images of dechorionated *Gammarus chevreuxi* embryos fixed at 14 dpf indicating, A) the position of the embryonic dorsal organ, B) the dorsal organ, C) the association between the dorsal organ and the chorion, and D) the position of the gills. DO and G indicate embryonic dorsal organ and gills respectively. Scale bars (10-100 µm) and magnifications (x230-1500) are shown for each image.

Figure 3. Light microscopy images of *Gammarus chevreuxi* embryos developed in the female marsupium. Embryos were stained with $AgNO_3$ at A) 6 dpf, B) 9 dpf, C) 14 dpf, and D) 18 dpf. Arrows with accompanying letter indicate silver stained areas, corresponding with the locations of the embryonic dorsal organ (DO) and gills (G). Scale bar = 200µm.

Figure 4. Osmolality of *Gammarus chevreuxi* homogenate of embryos removed from the mother at three different stages of development (i.e. pre-dorsal organ (pre-DO, dark grey), dorsal organ present (DO, dark blue), and gills present (Gill, light blue)) and subsequently exposed to salinities of 2, 15 and 33 for 24 h *in vitro*. Points represent mean concentrations and standard deviations of three biological replicates

566 per treatment, each consisting of pools of 25 embryos. Broken line represents the
567 isosmotic line.
568
569 Figure 5. Osmotic and ionic regulation in adult *Gammarus chevreuxi* acclimated to
570 four salinities (S= 2, 10, 25 and 35) for seven days. Haemolymph (A) osmolality (B)
571 sodium (Na^+) concentration, (C) calcium (Ca^{2+}) concentration and (D) magnesium
572 (Mg^{2+}) concentration. Points represent mean concentrations and standard errors of
573 pooled biological replicates from the respective treatments (n= 12, 15, 15 and 15 for
574 salinities 2, 10, 25 and 35 respectively (A); n= 40, 44, 40 and 19 for salinities of 2,
575 10, 25 and 35 respectively (B-D)). Broken lines represent isosmotic lines.
576
577 Figure 6. Expression levels (dCt) for two isoforms putatively identified as Na^+/K^+-
578 ATPase alpha subunit in four embryonic stages corresponding with <16-cells (<24
579 hpf), pre-dorsal organ (pre-DO, <48 h), dorsal organ present (DO, 9 dpf), gills
580 present (Gill, 18 dpf) and adults (*n* = 2-3 pools of 50 embryos per developmental
581 stage, and n= 3 pools of 10 adults). Transcripts are labelled ATPase alpha long
582 (transcript length= 4398 bp) and ATPase alpha short (transcript length= 4317 bp) for
583 convenience.
584

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